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Peripheralization of hemopoietic progenitors in primates treated with anti-VLA$_4$ integrin

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ABSTRACT **Interaction of hemopoietic cells with the elements of the underlying bone marrow stroma, the unique site of their "homing" in adult individuals, is essential for sustained normal hemopoiesis. However, the specific molecules responsible for homing and for the continuing interaction of hemopoietic cells with the bone marrow stromal cells *in vivo*, or those involved in progenitor/stem cell trafficking through the bloodstream, have not been defined. A large repertoire of adhesion receptors, especially of the integrin family, appear to play a prominent role in promoting adhesion of hemopoietic stem cells to cultured marrow stromal cells *in vitro*. To test the functional role of cytoadhesion molecules *in vivo*, we treated primates systemically with either anti-α_4- or anti-β_2-integrin antibodies, whose antigens are found in the majority of hemopoietic progenitors and in many differentiated cells. We found that anti-α_4 (anti-VLA$_4$, anti-CD49d) but not anti-β_2 (anti-CD18) treatment selectively mobilized progenitors into the bloodstream (up to 200-fold). Peripheralization involved erythroid, myeloid, and mixed progenitors; was detectable 24 hr after a single anti-VLA$_4$ injection; and lasted beyond the days of treatment. Anti-VLA$_4$ treatment additively augmented peripheralization of progenitors in animals with a preceding course of granulocyte-colony-stimulating factor. These data provide insight on the involvement of VLA$_4$ antigens in the *in vivo* trafficking of progenitors and are of relevance to collection of peripheral blood stem cells for transplantation.**

Cellular interactions between hemopoietic cells and their microenvironment (stromal cells and extracellular matrix) at specific tissue sites (e.g., bone marrow in adults) are important for their development and function (1–3). *In vitro* experiments exploring the adhesive interactions of hemopoietic progenitors with surrogate layers of bone marrow microenvironment have suggested that cytoadhesion molecules present in hemopoietic cells and the ligands or counter receptors for these molecules present in microenvironmental cells are responsible for this interaction (4–10). Thus, the VLA$_4$/vascular cell adhesion molecule (VCAM), the VLA$_5$/fibronectin, or the lymphocyte function-associated 1/intercellular adhesion molecule 1 (LFA-1/ICAM) pathways have been implicated, at least partially, in adhesive interactions with cultured bone marrow stroma. Antibodies to members of the respective pathways partially inhibited these interactions. Other molecules—i.e., selectins or heparan sulfate proteoglycans with unidentified ligands—have also been considered to participate in stem cell/stromal interactions (11, 12). Furthermore, treatment of murine bone marrow cells with antibodies to the common β_1 chain of VLA integrins, or to fibronectin, led to a decreased lodging of colony-forming units (CFUs) in murine spleens (13), whereas transplantation of murine bone marrow cells in the presence of synthetic neoglycoproteins with galactosyl and mannosyl specificity partially inhibited homing of stem cells to the marrow but to the spleen (14).

The significance, however, of the above observations the *in vivo* homing of stem cells to specific hemopoietic si or on the continuous interaction of progenitor cells with t stroma, and the continuous trafficking of progenitors thro the bloodstream, has been unclear.

To provide an insight into the *in vivo* functional rol some of the cytoadhesion receptors present in hemopoi cells, we initiated experiments in primates by treating t with anti-β_1 or anti-β_2 integrin antibodies. The antigens both of these are highly expressed in human and prin CD34$^+$ progenitor cells, as well as in differentiating cell several lineages (10, 15–17). With these treatments we ho to disrupt the normal homeostasis or the dynamic equilibr of circulating versus noncirculating progenitors. We fo that intravenous (i.v.) treatment of baboons or maca with saturating amounts of anti-VLA$_4$ antibody induc modest increase in circulating white cells, but a signific mobilization of hemopoietic progenitors of all classes. contrast, anti-β_2 integrin antibody treatment (anti-CD despite significant effects on peripheral neutrophil cou induced no peripheralization of progenitors. Furtherm anti-VLA$_4$ treatment subsequent to a 5-day course of g ulocyte-colony-stimulating factor (G-CSF) additively mented circulating progenitors. Although the mechanis of anti-VLA$_4$-induced mobilization of progenitors remain be determined, these data suggest that VLA$_4$ antigens involved in the *in vivo* trafficking of all classes of assay hemopoietic progenitors. Whether repopulating stem c are also mobilized, or whether VLA$_4$ antigens play a d nant role in the "homing" of transplanted stem cells, ca be determined without further experimentation.

MATERIALS AND METHODS

Animals. Healthy juvenile macaques (*Macaca nemestr* and baboons (*Papio cynocephalus anubis*) of either sex weighing 5–6 kg were housed at the University of Wash ton Regional Primate Research Center under approved ditions. Studies were approved by the institutional re board and by the Animal Care and Use Committee. animals were provided with food and water ad lib throughout the study. Blood samples and i.v. antibod jections were done under anesthesia, as described (18).

Monoclonal Antibodies for Immunofluorescence Stu Cells for antibody staining were prepared from antico lated macaque or baboon blood, lysed with ammo chloride buffer, and washed twice. Fluorescein- or ph erythrin-conjugated antibodies to CD16 (Leu-11a), (Leu-2a), CD69 (Leu-23), and CD4 (Leu-3a) were purch

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Abbreviations: BFUe, erythroid burst-forming unit; CFU, c forming unit; CFUe, erythroid CFU; GM-CFU, granulo macrophage CFU; G-CSF, granulocyte-colony-stimulating f GM-CSF, granulocyte/macrophage-colony-stimulating factor: interleukin *n*: VCAM, vascular cell adhesion molecule.

from [redacted]cton Dickinson. Directly conjugated anti-CD18 (60.3), anti-CD3 (FN18), anti-CD45RA (3AC5, anti-Lp220), anti-surface IgD (δ-TA4-1), anti-CD20 (1F5), and the MOPC11 control myeloma protein were all kindly provided by Edward A. Clark (University of Washington). Biotinylated monoclonal antibody 12.8 (anti-CD34) was kindly provided by CellPro (Bothell, WA), QBEND10 (anti-CD34) was purchased from AMAC (Westbrook, ME), and the streptavidin-phycoerythrin conjugate was purchased from Biomedia (Foster City, CA).

Progenitor-Cell Assays. Blood was collected in preservative-free heparin and the peripheral blood mononuclear cells were isolated by centrifugation on a cushion of Lymphoprep (Accurate Chemicals). Interface cells were washed and cultured in methylcellulose medium consisting of 0.9% methylcellulose (Fisher Scientific), 50% fetal bovine serum (Intergen, Purchase, NY), 1% bovine serum albumin (Calbiochem), and 0.1 mM 2-mercaptoethanol (Eastman Kodak) in Iscove's modified Dulbecco's medium (HyClone). Also included were erythropoietin (2 units/ml; Genetics Institute, Cambridge, MA), Kit ligand/stem-cell factor (SCF, 50 ng/ml; Amgen Biologicals), granulocyte/macrophage-colony-stimulating factor (GM-CSF, 50 ng/ml; Genetics Institute), and gibbon interleukin 3 (IL-3, 50 units/ml; kindly provided by Ken Kaushansky, University of Washington). All cultures were set up in duplicate or triplicate plates with cells plated at 100,000 or 500,000 per ml. After incubation at 37°C with 5% CO_2 at high humidity, erythroid burst-forming units (BFUe) and granulocyte/macrophage-colony-forming units (GM-CFU) were counted in plates of live cells on days 12–14 on the basis of morphologic criteria observed with a dissecting microscope. Macroscopically visible compact colonies (mixed or pure) which were over 0.5 mm or over 1 mm in diameter were counted separately in plates of live cells at day 21. Evaluation of erythroid colony-forming units (CFUe) containing eight or more cells and erythroid cluster-derived colonies (e-cluster) with two to eight cells was done in plasma clot cultures fixed at day 3 and stained with benzidine (19). The plasma clot medium contained 10% bovine embryo extract (GIBCO), 50% fetal bovine serum, 1% bovine serum albumin, 0.1 mM 2-mercaptoethanol, 10% bovine citrated plasma (Irvine Scientific) and the same amounts of growth factors as above.

***In Vivo* Treatments.** Endotoxin-free, protein A-purified anti-VLA_4 monoclonal antibody HP1/2 was a kind gift from Roy R. Lobb (Biogen) and was administered to four animals intravenously daily for 1 or 4 days at a dose of 1 mg/kg of body weight. The antibody, frozen in physiological buffer, was thawed, diluted in 0.9% NaCl with 0.1% human albumin (Armour Pharmaceutical) and given in a volume of 2–3 ml. Blood was drawn on one or two occasions before treatment, daily during treatment, and on several occasions after cessation of treatment. A fifth animal was treated with endotoxin-free anti-CD18 (60.3) antibody kindly provided by John M. Harlan (University of Washington). This was given in three single i.v. daily injections at 2 mg/kg of body weight in a volume of 5.9 ml. Two other animals were treated with a course of G-CSF (Filgrastim Neupogen, Amgen) for 5 days at 30 μg/kg of body weight per day divided into two daily subcutaneous doses, and one of these animals was subsequently treated for two days with the anti-VLA_4 antibody at 1 mg/kg each day. An eighth animal was treated with anti-VCAM-1 (4B9, kindly donated by John M. Harlan). This antibody is of the same isotype as HP1/2 and has weak reactivity with monkey endothelial cells.

RESULTS

Change in Hematologic Parameters Following Anti-VLA_4 Treatment. We treated three macaques and one baboon with anti-VLA_4 antibody HP1/2 by giving 1 mg/kg per day for 1 or 4 days intravenously. One macaque received one injection and the remaining three animals received four daily i.v. injections each.

Changes in white blood cell counts (lymphocytes or granulocytes) were determined on one or two occasions before treatment and were monitored daily after treatment for a period of 2 weeks. In each of the three animals receiving four injections, white cell count increased ≈2-fold and this increase included both granulocytes and lymphocytes (Fig. 1). No immature precursor forms of either myeloid (i.e., metamyelocytes, myelocytes) or erythroid lineages (e.g., nucleated red cells) were seen and no significant changes in hematocrit (except a small reduction most likely attributable to bleeding) and platelet counts were noted (data not shown). To test whether the increase in lymphocytes was due to a certain subset versus all classes of lymphocytes, we carried

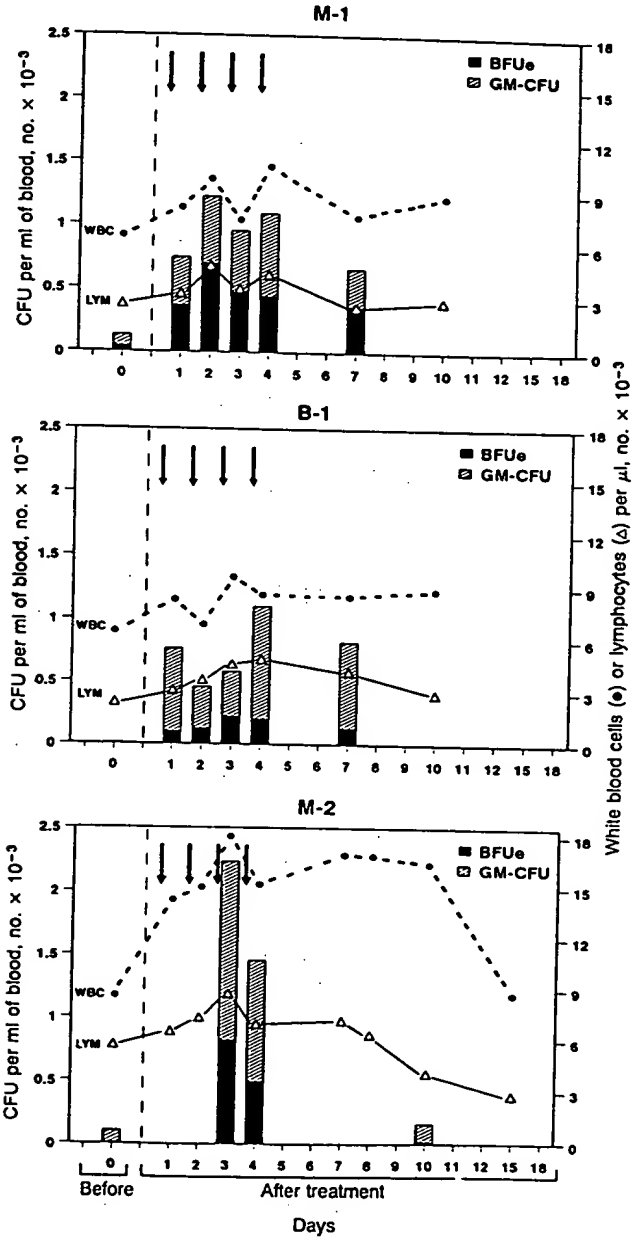

FIG. 1. Two macaques (M-1 and M-2) and one baboon (B-1) were treated with a daily i.v. injection of anti-VLA_4 antibody (HP1/2) at 1 mg/kg per day for 4 days (arrows). There was a moderate rise in the number of granulocytes and lymphocytes but an increase in total circulating CFU (per milliliter of blood) up to 200-fold.

out immunophenotyping using different anti-lymphocyte antibodies. As seen in Fig. 2, both T and B cells were increased over baseline levels, though it is possible that B cells were affected more than T cells. Effects on functionally specific subsets (i.e., $CD20^+IgD^-$, $CD20^+IgD^+$, $CD4^+CD45RA^+$, $CD8^+CD16^+$, etc.) were also evaluated by double-labeling experiments. No significant effects on a specific subset were noted (data not shown). The increase in total lymphocytes observed in our animals was much smaller than the ones observed previously in rats treated with anti-VLA_4 (20). The fact that saturating amounts of antibody were used is indicated in Fig. 3. After two injections of anti-VLA_4 there was no difference between control mononuclear cells (cells without the first antibody, i.e., without anti-VLA_4) and anti-VLA_4-treated cells (Fig. 3). Incubation of normal peripheral blood mononuclear cells from an untreated baboon with pretreatment plasma showed no labeling, whereas the same cells incubated with plasma 3 days after treatment with anti-VLA_4 showed labeling similar to that observed when anti-VLA_4 replaced the day 3 plasma (data not shown). These data also suggest that there is no significant modulation of VLA_4 antigen after anti-VLA_4 treatment.

Hemopoietic Progenitors Increase After Anti-VLA_4 Treatment. Progenitor cell assays were carried out before and on several occasions after treatment. For plating we used Lymphoprep-isolated cells and/or total nucleated cells present in a given volume of blood after red cell lysis. Quantitation of progenitors per milliliter of blood was estimated from the frequency of clonogenic progenitors in methylcellulose or plasma clot cultures and the total number of nucleated cells recovered either through Lymphoprep or after red cell lysis from a given volume of blood. Comparable results were obtained by both of these approaches. Before treatment, total assayable progenitors numbered from 10 to 100 per ml of blood; 24 hr after treatment and throughout treatment there was a significant increase up to 2200 per ml (Fig. 1). Specifically, GM-CFU increased 8- to 100-fold; BFUe increased 18- to >200-fold after treatment. Of interest, the size of colonies seen after treatment was also greatly increased so that macroscopically visible colonies of myeloid, erythroid, or mixed nature were present in significant numbers after treatment. This increase in total progenitors per milliliter of blood was largely attributed to an increase in the concentration of progenitors, from 6–11 total CFU per 10^5 plated cells before treatment to 86 progenitors per 10^5 plated cells seen after treatment. The macaque treated with a single injection of anti-VLA_4 showed an 8-fold increase in circulating progenitors 24 hr later.

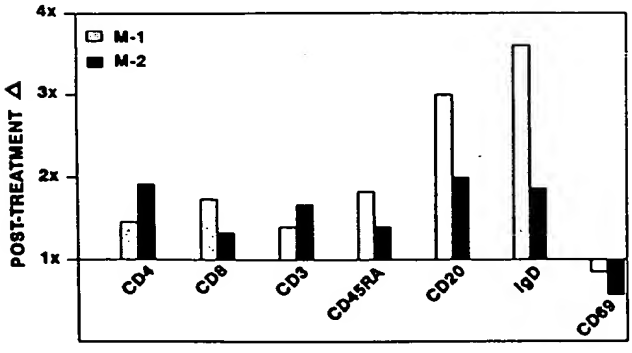

FIG. 2. Blood from the two macaques treated with 4 daily i.v. injections of anti-VLA_4 was lysed with ammonium chloride buffer, and the remaining cells were washed twice and stained with fluorescein- or phycoerythrin-conjugated antibodies on day 0 (pretreatment) and on day 3 or 4 after anti-VLA_4 treatment. After flow cytofluorimetric analysis, total numbers of labeled cells were calculated from the total lymphocyte count. Baseline pretreatment values are 1×. Note that all lymphocyte subsets, except CD69 (activated T lymphocytes), increased after anti-VLA_4 treatment.

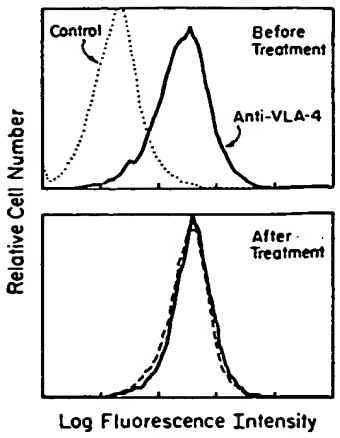

FIG. 3. (*Upper*) Relative fluorescence intensity profile of pretreatment baboon peripheral blood mononuclear cells labeled with fluorescein-conjugated anti-mouse $F(ab')_2$ alone (control) and with anti-VLA_4 (HP1/2) followed by fluorescein-conjugated anti-mouse $F(ab')_2$. (*Lower*) Mononuclear cells from the same animal after 2 days of anti-VLA_4 injections, stained as in *Upper*, show no difference between control and anti-VLA_4-treated cells.

Increase in Circulating $CD34^+$ Cells After Anti-VLA_4 Treatment. Circulating mononuclear cells drawn before treatment and once after treatment from three animals (the three animals in Fig. 1) were labeled with anti-CD34 (12.8 or QBEND10). Whereas $CD34^+$ cells were well below 1% before treatment, after treatment they comprised 1.9–3.5% of the cells in appropriately gated populations (data not shown).

Anti-β_2-Integrin (Anti-CD18) Treatment Does Not Mobilize Progenitors. To test whether the effect on progenitor peripheralization was specific to anti-VLA_4 monoclonal antibody or whether it could also be observed with antibodies to other integrins expressed in progenitor cells, we treated one macaque with saturating doses of anti-CD18 (60.3 antibody at 2 mg/kg per day) for 3 days and monitored the same parameters as with anti-VLA_4 treatment. With this treatment the expected increase in neutrophils was seen (Fig. 4) and this was ≈4- to 7-fold higher than that observed with anti-VLA_4. However, in contrast to VLA_4, no increase in lymphocytes and no increase in circulating progenitors were observed; the latter were unchanged when calculated per milliliter of blood, whereas there was a relative decrease in their concentration when calculated per 10^5 plated cells. One baboon was treated with two injections of anti-VCAM-1 (4B9 at 1 mg/kg). This antibody does not appear to react with baboon endothelial cells and no differences in either the white cells or the circulating progenitors were seen posttreatment, although saturating levels were reached (data not shown).

Anti-VLA_4 Further Augments G-CSF Mobilized Progenitors. *In vivo* treatments with various cytokines, including G-CSF (21–23), GM-CSF (24, 25), Kit ligand/stem-cell factor (26, 27), IL-1 (28), and IL-11 (29), have shown that they all are capable of peripheralizing progenitors, but with different potencies and under different kinetics. Although the mechanism(s) of cytokine-induced peripheralization of progenitors has not been elucidated, if overlapping mechanisms exist, one would not anticipate any additional mobilization by anti-VLA_4 treatment. To test this supposition, two baboons were treated subcutaneously with G-CSF for 5 days (30 mg/kg per day, divided in two injections). G-CSF was chosen because of its effectiveness and the significant background information already available. One animal was the control; the other was subsequently treated with anti-VLA_4. In both animals granulocytes immediately increased, so that 24 hr

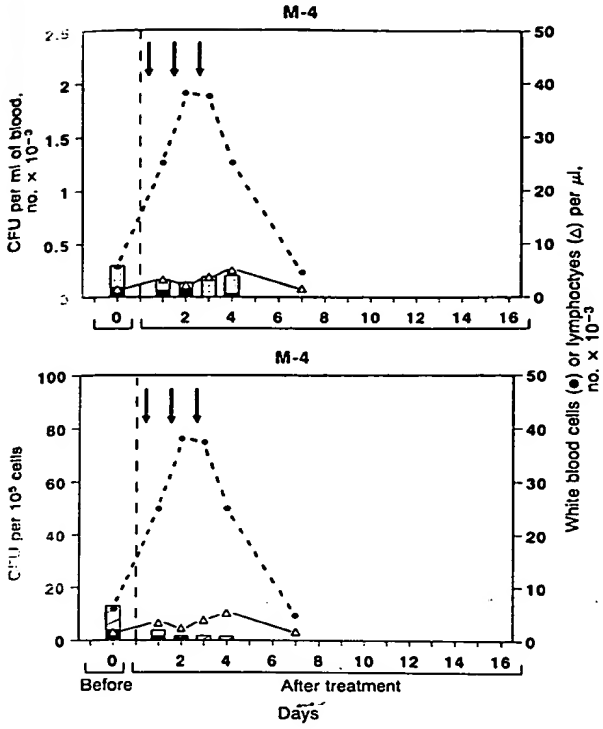

FIG. 4. One macaque (M-4) was given a daily i.v. injection of anti-CD18 (60.3 antibody) at 2 mg/kg per day for 3 days. There was an immediate increase in total white blood cells (●) but no increase in lymphocytes (Δ) or in total circulating CFUs (■, BFU-e; ⊡, GM-CFU).

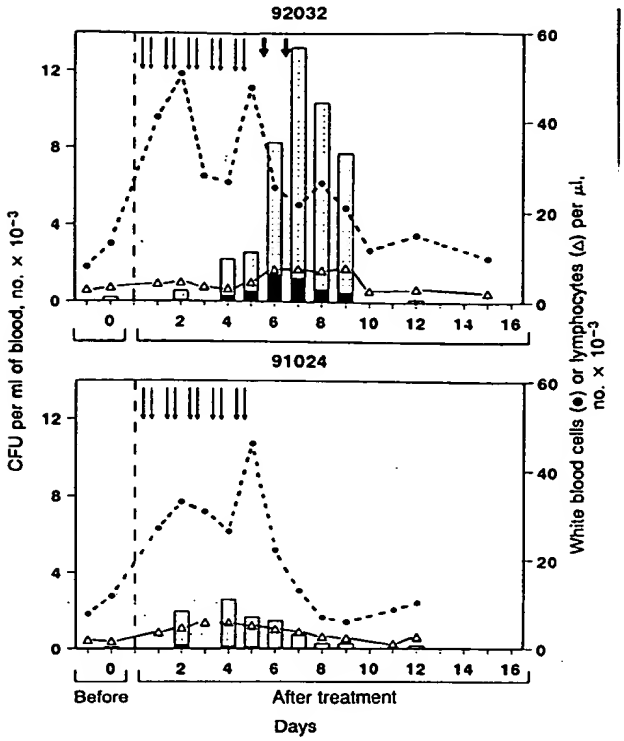

FIG. 5. Treatment of two baboons (92032 and 91024) with G-CSF (two daily subcutaneous injections, each at 15 mg/kg of body weight, for 5 days, as indicated by long arrows). One animal (92032, *Upper*) was given a follow-up treatment of anti-VLA$_4$ (HP1/2 at 1 mg/kg per day for 2 days, as indicated by short arrows). CFU per milliliter of blood showed a significant increase by days 4 and 5 of G-CSF treatment (11- to 14-fold above baseline). However, although white blood cells (●), lymphocytes (Δ), and levels of circulating CFU (■, BFUe; ⊡, GM-CFU) fell significantly after discontinuation of G-CSF, in the animal given HP1/2 following G-CSF the abrupt decrease in white blood cells was not seen and a progressive increase, instead of decrease (days 6–9), in CFU was observed.

later they were 3-fold above the pretreatment levels (Fig. 5). In addition there was an increase in progenitor number, from 89 per ml of blood pretreatment to 2500 per ml of blood posttreatment, or from 7 to >100 per 10^5 plated cells. As the white cell count decreased in the posttreatment period, there was a concomitant decrease in circulating progenitors in the control animal. However, in the animal that was treated subsequently with two doses of anti-VLA$_4$, there was a significant increase in progenitors beyond the level observed at day 5 of G-CSF (from 2000 to 13,000 per ml of blood). More importantly, this increase was maintained for ≈3 days after the last anti-VLA$_4$ treatment and occurred while white blood cells were decreasing. In contrast to G-CSF alone, there was a significant increase in BFUe, circulating CFUe, and nucleated red blood cells (Table 1). Furthermore, a significant number of macroscopically visible compact colonies (those above 0.5 mm in diameter) were observed only in the anti-VLA$_4$-treated animal.

DISCUSSION

Our data suggest that the VLA$_4$ antigen present in hemopoietic progenitors is involved in the trafficking of these progenitors through peripheral blood. Although the effect of anti-VLA$_4$ treatment on peripheralization of progenitors seems to be specific, the mechanism(s), direct or indirect, through which this effect is exerted is unclear. Several possibilities can be entertained alone or in combination. (*i*) Detachment or dislodgment of (not firmly attached) progenitors from marrow stroma. If the main function of VLA$_4$ is to stabilize the interaction of progenitors with their stroma, the presence of anti-VLA$_4$ would interfere with their stabilization. (*ii*) Blocking newly generated progenitors from attaching to marrow stroma. Antibody-coated progenitors would no longer be able to attach themselves to stroma. (*iii*) Interference with tissue (other than bone marrow) distribution of circulating progenitors. Redistribution of progenitors temporarily residing in other organs such as liver or spleen could be affected. (*iv*) Indirect effects of anti-VLA$_4$ treatment through recruitment of other pathways—i.e., cytokine increases or increase in soluble VCAM. As VLA$_4$ is present in many accessory cells, antibody treatment could affect signaling

Table 1. Progenitor concentration per ml of blood in G-CSF control baboon (C) and the baboon treated with both G-CSF and anti-VLA$_4$ (T)

	Total CFU		Macro-CFU		CFUe		e-clusters*	
Day	C	T	C	T	C	T	C	T
0	88	194	0	0	0	0	0	0
[2	1957	542	7	0	48	0	294	2,632
4	2580	2,224	20	28	42	284	550	9,590
→5	1644	2,604	6	113	8	115	396	1,209
→6	1465	8,314	0	664	5	1558	42	30,114
7	743	13,255	1	503	0	1966	14	17,446
8	270	10,402	0	190	0	97	0	4,030
9	256	7,261	0	154	0	521	0	7,508
12	136	151	0	0	0	0	0	6

Bracket indicates days of G-CSF treatment; arrows indicate the two injections of anti-VLA$_4$.

*Aggregates of two to eight erythroblasts present 3 days after plating in clot cultures.

pathways in these cells, which could lead to elaboration of specific cytokines. Direct testing of some of the above possibilities may not be possible. Nevertheless, several indirect approaches to dissect some of these possibilities could be pursued. For example, use of nonblocking versus attachment-blocking anti-VLA_4 antibody or use of anti-primate VCAM-1 antibody, if available, could provide useful insight. Furthermore, the fact that anti-VLA_4 was highly effective in inducing mobilization of progenitors generated under G-CSF treatment, a valuable but indirect approach, suggests that the VLA_4 pathway may not be a dominant one in G-CSF-induced progenitor mobilization or that an indirect elaboration of G-CSF-induced by anti-VLA_4 treatment is not of significance.

It is intriguing that precursor cells, either erythroid or myeloid (i.e., erythroblasts, myelocytes), are not mobilized by anti-VLA_4 treatments, in contrast to progenitor cells, although both express the VLA_4 antigen. This may reflect differences in the state of affinity of VLA_4 between these two classes of cells. Alternatively, differences in cytoskeletal proteins between early progenitors and precursors could influence peripheralization, if these proteins participate in adhesive interactions. Furthermore, whether the basis of the increase in peripheral lymphocytes is the same as that for progenitor peripheralization remains to be substantiated. Involvement of VLA_4 in lymphocyte trafficking has been previously substantiated in the rabbit model, in which a more exaggerated response has been noted (20). Likewise, whether VLA_4 is involved in homing and/or engraftment of multipotent stem cells remains to be seen.

It is of interest that an antibody to a specific integrin mobilized a wide range of progenitor cells, including progenitors with high proliferative potential *in vitro*. Furthermore, given the additive effect on the G-CSF-pretreated animal, our results suggest a useful means to boost progenitor peripheralization and to properly time apheresis sampling for transplantation, provided that engraftment is unimpaired by anti-VLA_4 treatment.

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